Annual progress Report on research related to our research project "Stabilization of Plutonium in Subsurface Environments via Microbial Reduction and Biofilm Formation" funded by the Environmental Remediation Sciences Division (ERSD).

Principal Investigator:

Dr. Mary Neu
Actinide Environmental and Coordination Chemistry
Los Alamos National Laboratory, Los Alamos, NM 87545
505-667-7717 (phone)
505-667-9905 (fax)
mneu@lanl.gov (email)

1. Research Objective. The overarching goal of this research project is to investigate and optimize the mechanisms for *in situ* immobilization of Pu species by naturally-occurring bacteria. Specific research objectives are: a) investigate the mechanism of bacterial accumulation and immobilization of plutonium species by biofilm formation under aerobic conditions and b) to demonstrate the direct and indirect stabilization of Pu via dissimilatory reduction by *Geobacter metallireducens*.

2. Research Progress and Implications. This section briefly summarizes results of studies after two years of a three-year project. The first part examines Pu(VI), Pu(V), colloidal $Pu(IV)(OH)_4(am)$, Pu(IV)(EDTA) and Np(V) reduction by metal reducing bacteria *Geobacter metallireducens* GS15 and *Shewanella oneidensis* MR1 and describes the implications of the findings for the potential remediation of Pu and mixed Pu, U sites. The second section summarizes work performed during the second year on the immobilization of uranium and plutonium by biofilms of *P. putida*.

2.1 Pu and Np reduction by metal reducing bacteria.

2.1.1 Plutonium(VI) and Pu(V) reduction by metal reducing bacteria *Geobacter metallireducens* GS15 and *Shewanella oneidensis* MR1. Bacterial reduction of actinides emerges as one of the most promising environmental processes that may be used to stabilize actinide contaminants in subsurface environments. Metal reducing bacteria have been shown to stabilize uranium and technetium and a number of other metals. The bioreduction of the mobile oxidized species such as U(VI) produces mineralized aggregates of highly insoluble and potentially immobile biogenic materials. The behavior of plutonium with regard to bacterial reduction is not well understood. Plutonium has a wider redox range and the speciation of its reduced forms is more complicated than for other actinides because its tetravalent oxidation state is accessible to reduction.

We examined the ability of metal reducing bacteria *Geobacter metallireducens* GS15 and *Shewanella oneidensis* MR1 to reduce Pu(VI), Pu(V) colloidal $Pu(IV)(OH)_4(am)$ and Np(V) and $Pu(IV)(OH)_4(am)$ under cell suspensions conditions.

Pu(VI) and Pu(V) are soluble at neutral pH but in the presence of chelators and bacterial cells these species tend to reduce rapidly to Pu(IV). The data in figure 1 show the changes in total aqueous Pu(VI)/Pu(V) concentrations during the cell suspension experiments performed to examine Pu(VI) and Pu(V) reduction by *G. metallireducens* GS15 and *S. oneidensis* MR1. The experimental cultures for both *G. metallireducens* GS15 and *S. oneidensis* MR1 with live cells and electron donor show the most rapid decrease in soluble Pu concentrations (Pu(V) and Pu(VI)) relative to the control cultures (Figure 1 A and B). There were no significant changes in soluble Pu concentrations in the controls with no cells and with heat-killed cells. The control with live cells and electron donor also show a decrease of soluble Pu indicating an active reduction process. By the end of the experiment most of the added Pu(VI) was present as Pu(V) in the controls without cells or with dead cells. However the soluble Pu concentration remained unchanged because Pu(V) is soluble. In the experimental cultures with live cells all initial Pu(VI) was reduced to Pu(IV).

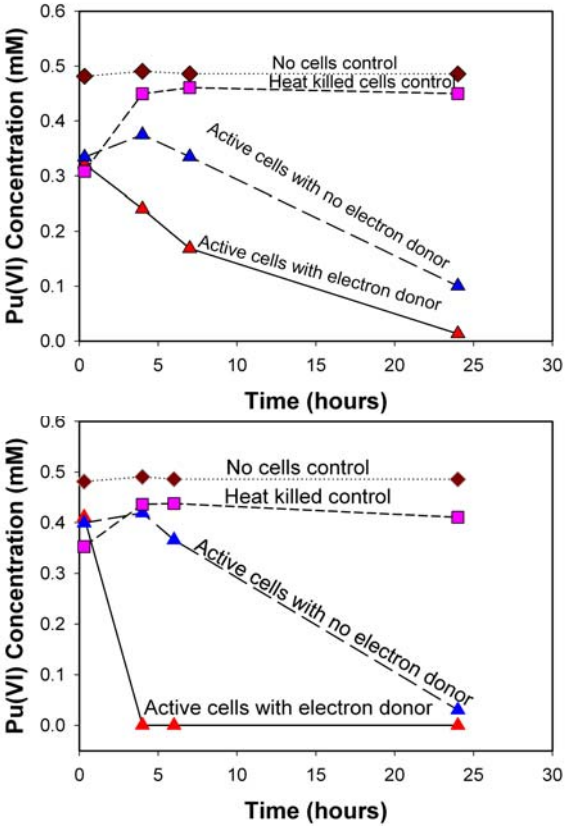

Figure 1. Direct reduction of Pu(VI) by cell suspensions of *Shewanella Oneidensis MR1*(A) *and Geobacter metallireducens* GS15 (B). Conditions: Cell density = 5×10^8 cells/mL suspended in 100 mM MOPS at pH = 7.4, [Pu(VI)] = 0.50 mM, T = 30 °C. The concentration of the electron donor were [Acetate] = 10 mM for S. *Oneidensis MR1(A)and* [Lactate] = 10 mM for *Geobacter metallireducens* GS15 (B). (▲) Live cells with the electron donor, (△) live cells with no electron donor, (◇) no cells control, (□) heat killed cells control

We characterized the solids formed using defuse reflectance and transmission electron microscopy imaging (TEM). The defuse reflectance spectra of colloidal Pu(IV) obtained by precipitation of Pu(IV) at near neutral pH and bioreduction solids obtained by incubation of 0.5 mM Pu(VI) with a cell suspension of *S. oneidensis* MR1 are very similar and strongly suggest that the reduction product from our experiments is indeed colloidal Pu(IV) (data not shown). The TEM images obtained by visualization of the solids obtained from the bio-reduction of Pu(VI) by *S. oneidensis* MR1 are shown in Figure 2. We have also obtained TEM images of U(VI) solids obtained following U(VI) reduction by *S. oneidensis* MR1 for comparison (data not shown). The images obtained for both Pu and U show the precipitation of actinides on the cell surface in addition to aggregates of solids dispersed between the cells. The TEM images of an expanded view of the Pu solids deposited near the cell surface

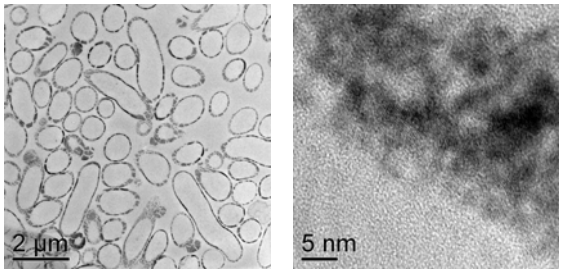

Figure 2. TEM image biogenic Pu(IV) solids obtained by bioreduction of Pu(VI) by cell suspension of *S. Oneidensis*(left) and an expanded view of cell wall area showing the crystalline morphology of the Pu solids (right).

(figure 2 right) show aggregates of nanoparticules of crystalline Pu(IV) deposited on the surface of the cells.

2.1.2 Np(V) reduction by metal reducing bacteria *Geobacter metallireducens* GS15 and *Shewanella oneidensis* MR1. We examined the ability of metal reducing bacteria *Geobacter metallireducens* GS15 and *Shewanella oneidensis* MR1 to reduce neptunium(V) and neptunium(V)-citrate. The toxicity of Np(V) to these organisms was also examined under growth conditions in a Fe(III)-citrate growth medium. Growth was significantly inhibited (a toxic effect) with Np(V)-citrate concentrations of 4 mM and greater for both bacteria. No inhibition was observed when Np(V)-citrate concentrations were 2 mM or less, and both Fe(III) and Np(V) were rapidly reduced. Cell suspensions of *S. oneidensis* were able to directly reduce unchelated Np(V) to insoluble $Np(IV)_{(s)}$; however, cell suspensions of *G. metallireducens* were unable to reduce unchelated Np(V). The addition of citrate as a complexing agent for Np(V) led to an enhanced Np(V) reduction rate by *S. oneidensis* and an observable reduction rate by *G. metallireducens.* However, growth was not observed for either organism when unchelated Np(V) or Np(V)-citrate were provided as the sole electron receptors. During Np(V)-citrate reduction, the reduced form of neptunium remained soluble, presumably as a poly-citrate complex.

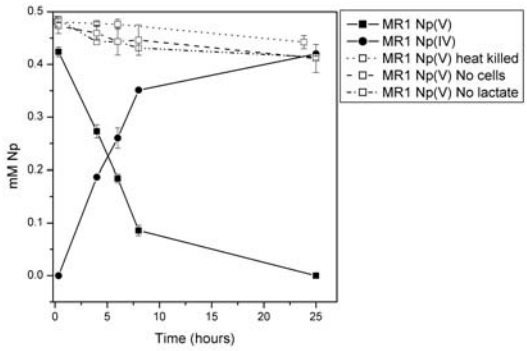

Figure 3. Direct reduction of Np(V)-citrate by a cell suspension of *S. oneidensis*. Conditions: Cell density = 4×10^8 cells/mL suspended in 100 mM MOPS, [Np(V)] = 0.50 mM, [citrate] = 50 mM, T = 30 °C.

2.1.3 $Pu(IV)(OH)_4(am)$ reduction by metal reducing bacteria *Geobacter metallireducens* GS15 and *Shewanella oneidensis* MR1. We examined the ability of these metal reducing bacteria to reduce freshly precipitated $Pu(IV)(OH)_4(am)$ under cell suspension conditions and examined the effect of chelating ligands on this process. Cell suspensions of metal reducing bacteria were found to reduce minimal amounts of $Pu(IV)(OH)_4(am)$. *Shewanella oneidensis* MR1 is slightly more efficient and reduces about 8 % of total plutonium added compared to less than 1 % for *Geobacter metallireducens* GS15. In the presence of one equivalent of EDTA both *Shewanella oneidensis* MR1 and *Geobacter metallireducens* GS15 completely reduced and dissolved $Pu(IV)(OH)_4(am)$. The concentration of Pu(III) in the cultures was followed by UV-visible spectroscopy and by scintillation counting of the amounts of soluble Pu. The data in figures 3 A and B show the evolution of Pu(III) concentration over time determined spectrophotometrically. In order to understand the mechanism by which complexants like EDTA enhance bacterial ability to reduce and solubilize Pu hydrous oxides, we investigated the reduction of soluble Pu(IV)(EDTA) by cell suspensions of these bacteria. We found that the apparent rate of Pu(IV)(EDTA) reduction (~ hours) is significantly faster than the apparent rates observed with other actinides (~ days). Both bacteria examined here were unable to use any of the plutonium forms tested as an electron acceptor to support their growth.

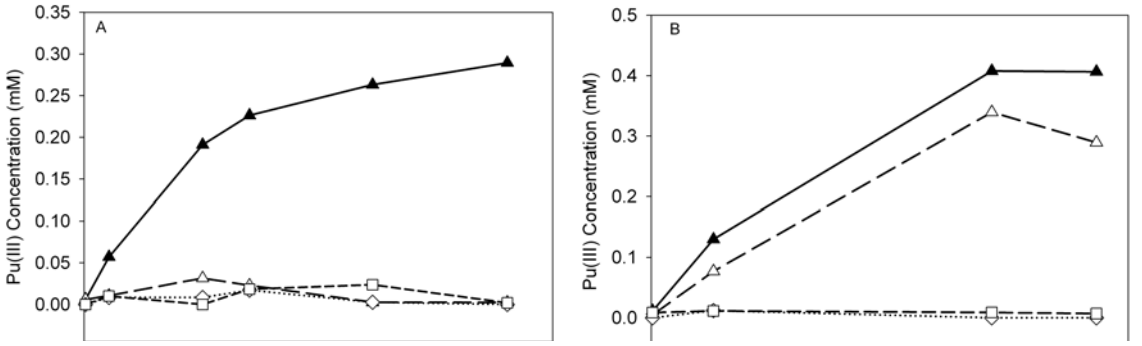

Figure 4. Direct reduction of $Pu(OH)_4(am)$ by cell suspension of *Shewanella oneidensis* MR1 (A) and *Geobacter metallireducens* GS15 (B) with 0.5 mM EDTA. Conditions: Cell density = 5×10^8 cell/mL, pH = 7.40, (A) [Lactate] = 10 mM, (B) [Acetate] = 10 mM $[Pu(OH)_4(am)]$ = 0.50 mM. (▲) Live cells with the electron donor, (△) live cells with no electron donor, (◇)no cells control, (□) heat killed cells control.

2.1.4 Implications of plutonium reduction for the remediation of Pu and mixed Pu, U sites. Microbial transformation of Pu hydrous oxides through direct and indirect reduction can have dramatic consequences on the fate and transport of Pu in the environment. Pu contamination present in the environment is mainly present as insoluble Pu(IV) hydrous oxides or associated to colloids and its transport occurs mainly under these forms. However, if Pu solids are present under anaerobic conditions and under active bio-reduction conditions, the concentrations of soluble Pu can dramatically increase. The presence of complexants, which are ubiquitous in biofilms, could completely change the mode of Pu transport. Metal reducing bacteria examined here were not able to use Pu species to support their growth and the reduction of $Pu(IV)(OH)_4(am)$ was inefficient in the absence of complexants, but environmental factors can significantly affect this process. Biogenic chelators are abundant in the environment and some microorganisms produce large amounts of complexant as a reaction to contaminants or for nutrient acquisition. For example bacteria and fungi commonly produce siderophores for nutrient iron acquisition. Other organic complexing agents such as polysaccharide, organic acids, amino carboxylic acids, and other natural organic chelators are common is the environment. These ligands can play an important role in mobilizing plutonium.

Under environmental conditions, it is unlikely that the concentration of Pu species would be sufficiently high to sustain growth of metal reducing bacteria. However, most microorganisms use Fe(III), Mn(IV), sulfate or nitrate species that are present in almost all soil environments to support their growth. It is therefore possible that indirect reduction of Pu species by reduced forms of Mn, Fe, or any reactive reduced substrates produced by these organisms would have a big impact on Pu reduction and subsequent solubilization. It is not known how Pu(III) would interact with various mineral surfaces and its stability in the environment is also unknown especially in the presence of complexants. We have demonstrated that Pu(III) can be produced by direct enzymatic reduction but a lot remains to be done to fully understand the consequences of Pu hydrous oxides reduction on the fate and transport of Pu in the environment. The implications of Pu(IV) reduction for possible Pu mobilization at sites containing mixed Pu, and U contamination needs further fundamental understanding, especially if

complexants are present. A recent investigation of the stability of U(IV) species under anaerobic conditions in the presence of the natural siderophore desferrioxamine B showed that the complex U(IV)(desferrioxamine B) remained soluble. This emphasizes the importance of bacterial metabolites on the fate and transport of actinide species in the environment.

2.2 Bacterial accumulation and immobilization of plutonium and uranium species by *P. putida* biofilms.

2.2.1 Effect of uranium and plutonium species on *P. putida* biofilm growth.

The fate of actinide species adsorbed onto mineral species under the action of microbial processes and biofilm growth are relatively unknown. To investigate the effect of U and Pu species on biofilm growth and the subsequent effect of biofilms on actinides we cultured *P. putida* biofilms on solid agar media and varied the growth conditions and Pu and U content. We evaluated the toxicity effect of the species examined on biofilm growth and the immobilization of Pu and U within biofilms. Biofilms of *P. putida* were grown on a solid agar growth media containing either unchelated uranium, U(VI)(EDTA), Pu(IV)(EDTA), or EDTA. Biofilm growth was significantly inhibited at Pu and U concentrations of 2.0×10^{-4} M for Pu and 4.0×10^{-4} M for uranium. No inhibition was observed when Pu and U concentrations were less than 1.0×10^{-4} M. All of the following experiments were performed at U and Pu concentrations of 1.0×10^{-4} M which did not show significant growth inhibition (Figure 5).

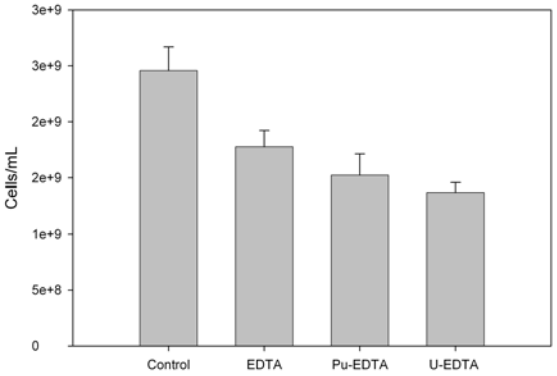

Figure 5. Growth of *P. putida* biofilms on agar media with no contaminant, 10^{-4} M of EDTA, 10^{-4} M Pu(IV)(EDTA) and 10^{-4} M U(VI)(EDTA).

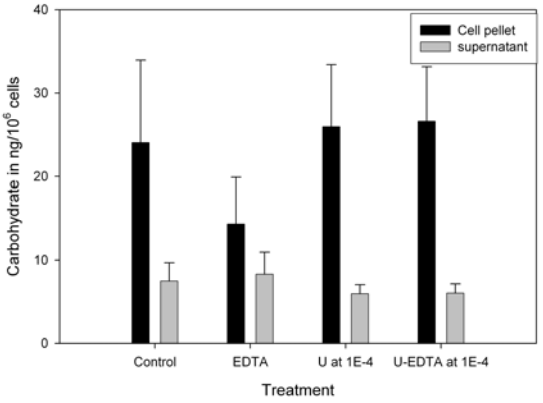

Figure 6. EPS production by biofilms exposed to 10^{-4} M U.

We examined the effects of Pu and U species on the production of extracellular polymeric substances (EPS) and found that under the concentrations used (1.0×10^{-4} M) the levels of EPS production remained comparable to controls (Figure 6).

We also examined actinide uptake by biofilms and found that under similar concentrations, Pu accumulation was substantially higher than that of Uranium (figure 7). Plutonium taken up by the biofilms was associated mainly with the cells. No plutonium was detected in the supernatant suggesting that Pu is transported inside the cells or tightly bound to the cell membrane. Uranium associated mostly to the cells, but a significant amount remained in the soluble fraction of the biofilms and is attributed to the solubilization by bacterial metabolites such as siderophores, EPS and other unidentified metabolites.

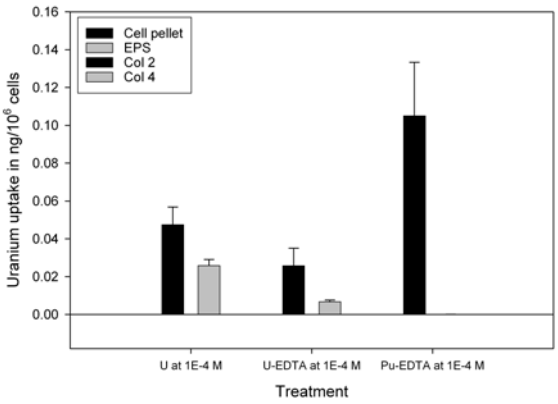

Figure 7. Plutonium and uranium uptake by *P. putida* biofilms. The concentrations of U and Pu were set to 1.0 10^{-4} M.

3. Planned Activities. In the last year of the project we will complete the experiments on the reduction of Pu by *G. metallireducens*. We have found that Pu(VI), Pu(V), and colloidal $Pu(IV)(OH)_4(am)$ were all accessible to bacterial reduction and we have characterized the reduction products. We will examine the accessibility of different PuO_2 forms to bacterial reduction and investigate the mechanism(s) and rates of Pu reduction by DMRB. We will prepare a manuscript for publication of the results on PuO_2 reduction. We will prepare a manuscript using the results from the biofilm studies and will complete biofilm studies using Pu and U sorbed onto mineral phases and prepare the results obtained for publication.

4. Information Access.

Publication list.

Boukhalfa, H., Icopini, G.A., and Neu, M. P., (2006) "Pu(IV) Reduction by Metal Reducing Bacteria *Geobacter metallireducens* GS15 and *Shewanella oneidensis* MR1", In preparation for *Environmental Science and Technology*.

Icopini, G.A., Boukhalfa, H., Reilly S.D., and Neu, M.P.,(2006) "Pu(V)/(VI) Reduction by Metal Reducing Bacteria (in preparation), In preparation for *Environmental Science and Technology*.

H. Boukhalfa, S.D. Reilly, and M. P. Neu (2006) "Siderophore-Mediated Transport of Metals into *Pseudomonas putida*: Insights into the Mechanism of Iron and Radionuclide Accumulation by Environmental Bacteria. (paper in preparation).

G. A. Icopini, H. Boukhalfa, and M. P. Neu (2006) "Biological Reduction of Np(V) and Np(V)-Citrate by Metal Reducing Bacteria" *Environmental science and technology*. In press.

H. Boukhalfa, S.D. Reilly, and M.P. Neu (2006) "Complexation of Pu(IV) with the natural siderophore desferrioxamine B and the redox properties of Pu(IV)siderophore complexes" *Inorg. Chem*. In press.

H. Boukhalfa, S.D. Reilly, R. Michalczyk, S. Iyer, and M.P. Neu (2006) "Iron(III) Coordination Properties of a Pyoverdin Siderophore Produced by *Pseudomonas putida* ATCC 33015" *Inorg. Chem.* (2006); *45(14);* 5607-5616.

M. P. Neu, G. A. Icopini, H. Boukhalfa (2005) "Plutonium Speciation Affected by Environmental Bacteria" *Radiochimica Acta, 93*, 705-714.

G. A. Icopini, H. Boukhalfa, and M. P. Neu (2005) "Environmental reduction of Tc, U, Np, and Pu by bacteria and the stability of reduction products". *In* Recent Advances in Actinide Sience, Royal Society of Chemistry, London, p 20-25.